



Attenuation of fibroblast growth factor signaling by poly-N-acetyllactosamine type glycans

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ABSTRACT

Fibroblast growth factors (FGFs) and their receptors are expressed in a variety of mammalian tissues, playing a role in development and cell proliferation. While analyzing human sperm motility, we found that sperm treated with endo- β -galactosidase (EBG), which specifically hydrolyzes poly-N-acetyllactosamine type glycans (polyLacs), enhanced motility. Mass spectrometry analysis revealed that sperm-associated polyLacs are heavily fucosylated, consistent with Lewis Y antigen. Immunohistochemistry of epididymis using an anti-Lewis Y antibody before and after EBG treatment suggested that polyLacs carrying the Lewis Y epitope are synthesized in epididymal epithelia and secreted to seminal fluid. EBG-treated sperm elevated cAMP levels and calcium influx, indicating activation of fibroblast growth factor signaling. Seminal fluid polyLacs bound to FGFs in vitro, and impaired FGF-mediated signaling in HEK293T cells.

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1. Introduction

Poly-N-acetyllactosamine type glycans (polyLacs) are large carbohydrates composed of galactose and N-acetylglucosamine repeats. PolyLacs were specifically detected due to their susceptibility to endo- β -galactosidase (EBG) [1–6]. They often carry blood group or onco-developmental antigens [3,7–9], which are detected by specific monoclonal antibodies directed to these carbohydrate antigens. PolyLacs and their modified forms are recognized as antigens, i.e., sialyl Lewis X and Y. These antigenic glycans reportedly function in cell–cell interaction during embryogenesis, tumorigenesis and cancer metastasis [10–12].

Because of their large size, polyLacs protect core glycoproteins from proteolysis [13]. Two lysosomal membrane proteins, LAMP1 and LAMP2, are heavily glycosylated with polyLacs [14]. Mice deficient in *Lamp2* accumulate autophagic vacuoles in many tissues

and die prematurely of cardiomyopathy [15]. PolyLacs are likely essential for survival of mammalian cells in vitro, since no polyLac-deficient cell line has been generated. PolyLacs are synthesized by sequential activity of B4GALT1 plus either B3GNT2 [16–19], B3GNT4 [18], B3GNT7 [20,21], and B3GNT8 [16]. *B3gnt2* knockout mice exhibit shorter polyLac chains than do wild-type mice, and their T-lymphocytes exhibit increased calcium flux and cell proliferation, suggestive of hyperactivation [16]. There are as yet no reports of mutant mice completely lacking polyLacs.

Previous studies, including our own, suggested that mature human sperm cells are densely covered by carbohydrates [22,23]. While we attempted to remove glycans from living human spermatozoa using glycosidases, we found human sperm cells treated with endo- β -galactosidase (EBG) moved more rapidly than untreated sperm. We investigated the mechanism underlying EBG-triggered sperm motility and found that seminal fluid polyLacs down regulate sperm motility by impairing fibroblast growth factor-dependent signaling.

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2. Materials and methods

2.1. Reagents

EBG from *Escherichia freundii* was obtained from Seikagaku Co. Ltd. (Tokyo Japan) and also purified from culture medium of *E. freundii*, as described [24]. Bovine corneal keratan sulfate was prepared as described previously [4,25]. Monoclonal anti-Lewis Y antibody (clone AH6, mouse IgM) [26] was provided by Dr. S. Hakomori, University of Washington, Seattle, WA. Recombinant human FGF1 (acidic-FGF) and FGF2 (basic-FGF) were obtained from Invitrogen/Life Technologies.

2.2. Human sperm and seminal fluid samples

Sperm and seminal fluid samples were obtained for future research use from 20 study volunteers, who provided verbal informed consent to participate in this study. Written consent was not obtained, as semen sample collection involves minimum health risk to human subject. Samples were given number codes to protect donor identity, and records were kept in a secure computer not accessible under any condition including by the internet. The ethics committee of the Hamamatsu Red Cross Hospital and that of Hamamatsu University School of Medicine approved the consent procedure. Donor fertility was not tested, nor did we undertake normal semen analysis. Sperm was mixed with sperm freezing solution from Irvine Scientific (Santa Ana, CA) and frozen until analyzed.

2.3. Sperm motility analysis

Sperm motility was analyzed using a SQA-V, sperm quality analyzer-V [27] (Medical Electronic Systems). Cryopreserved human sperm from healthy volunteers were washed with sperm washing medium (Irvine Biologicals) and suspended in the same solution. EBG was dissolved in water at 1 mUnit/ml. To 100 μ l of human sperm solution (2×10^6 cells), 5 μ l EBG was added at final concentration 50 μ Units/ml, and incubated at room temperature for 15 min. A sperm aliquot was placed in a 500 μ l SQA-V Measurement Capillary. Statistical analysis was performed using a paired two-tailed Student's *t*-test using the Prism program (GraphPad Software).

2.4. Measurement of calcium influx and cAMP

Calcium influx into sperm cells was measured using a Fluo-4 NW calcium assay kit (Molecular Probes). Sperm cells treated or untreated with EBG were assayed in triplicate in 96-well plates. Fluorescence was measured using a Beckman DTX810 plate reader, using excitation and emission wavelengths of 495 and 519 nm, respectively. Cyclic AMP was measured using a competition-based immunoassay kit for cyclic AMP and GMP XP (Cell Signaling).

2.5. Preparation of sperm-associated glycans and mass spectrometry analysis

Upon thawing, sperm cells were collected by centrifugation at $400 \times g$ for 5 min, washed twice with phosphate buffered saline, and digested with 20 μ l Proteinase K (14–22 mg/ml, Roche) at 45 °C for 20 h. After removing insoluble materials by centrifugation, supernatants were treated with 0.5 M NaOH containing 1 M NaBH₄ at 37 °C for 20 h. Samples were passed through Sephadex G-25 equilibrated with water, and materials eluting in the void volume were pooled and applied to a Sephadex G-50 super fine column equilibrated with 0.2 M NaCl. Neutral sugars were

monitored by the anthrone color reaction [28], and glycopeptides carrying large glycans or polyLacs were collected for mass spectrometry analysis. The sample was permethylated for MALDI-MS and MS/MS analyses using conditions as described for recovering both non-sulfated and sulfated glycans [29].

2.6. Preparation of seminal fluid polyLacs

Human seminal fluid was prepared from a pool of samples. Semen was mixed with three volumes of chloroform/methanol (2:1, v/v) to extract lipids. After centrifugation, the pellet was suspended with 0.1 M Tris–HCl buffer, pH 8.0, containing 1 mM EDTA, and digested by protease K (20 μ g/ml) at 45 °C for 20 h. After centrifugation at $12000 \times g$ for 30 min, water soluble materials were passed through Sephadex G-25 equilibrated with water, and materials eluting in the void volume were applied to a Sephadex G-50 super fine column as described above. PolyLacs were collected and de-salted using a Sephadex G-25 column and lyophilized.

2.7. Immunohistochemistry

Paraffin-embedded sections of human testis, epididymis, and ductus deferens tissues were obtained from Folio Biosciences (Columbus, OH). After deparaffinization, hydration and peroxide-treatment, one of each paired slides was subjected to EBG treatment at 37 °C for 30 min. Tissue specimens were stained by anti-Lewis Y (clone AH6) antibody [26], followed by biotinylated goat anti-mouse IgM antibody (Vector) and peroxidase-conjugated streptavidin. The peroxidase color reaction was performed using DAB substrate on paraffin-embedded tissue sections or AEC single solution (Invitrogen) on sperm smears, and all tissues were counterstained using hematoxylin. Unwashed and PBS-washed human sperm cells were smeared on glass slides, air-dried and fixed with 4% paraformaldehyde in PBS. Tissues were then treated with or without EBG and immunostained as described above for tissue sections.

2.8. FGF plate binding assay

Human recombinant FGF1 or FGF2 (0.5 μ g) was diluted in 100 μ l water and added to wells of 96-well polyvinylchloride flat bottom plates (Coster), which were then incubated 20 h at 4 °C. After washing wells with PBST (0.2 M Na-phosphate buffer, pH 7.4, containing 0.2% Tween 20), wells were blocked with Superblock 20 (Pierce). PBST (50 μ l) with or without heparin (200 μ g/ml) was added to each well and incubated for 15 min at room temperature. Seminal fluid polyLacs (200 μ g/ml in PBST, 50 μ l) was then added to each well and incubated for 15 min. After washing with PBST, diluted (1:3000) anti-LeY antibody (AH6) [26] was added to wells and reacted at room temperature for 60 min. After washing with PBST, diluted (1:2000) peroxidase-conjugated anti-mouse IgM antibody was added and reacted at room temperature for 30 min. After washing with PBST, wells were reacted with TBM substrate solution (eBioscience) at room temperature for 30 min for peroxidase-based color reaction. The color reaction was stopped by addition of 2 N-sulfuric acid, and absorbance at 450 nm was measured in a plate reader.

2.9. Glycan arrays

Glycan microarrays were printed as described [30]. For the analysis, v5.1 (www.functionalglycomics.org/static/consortium/researches/resourcecoreh8.shtml) was used. Recombinant FGF2 was diluted to 200 μ g/ml in TBSCM (20 mM Tris, 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, pH 7.4) containing 1% bovine serum albumin

and 0.05% Tween-20, with or without 100 $\mu\text{g/ml}$ heparin. A 70 μl aliquot was applied to a microarray slide and incubated for 60 min under a coverslip in a humidified chamber at room temperature. Coverslips were then gently removed in a solution of TBSCM/0.05% Tween-20 and subjected to the TBS standard wash procedure. To detect protein binding, the slide was similarly incubated with mouse monoclonal anti-FGF2 antibody (clone bFM2, Millipore) and washed as described. The slide was then incubated in the dark with anti-mouse IgG antibody labeled with Alexa 488 (Qiagen) at 5 $\mu\text{g/ml}$, washed as described above, and then washed with deionized water to remove salts. The slide was then spun in a slide centrifuge for approximately 15 s to dry and immediately scanned in a PerkinElmer ProScanArray Scanner using an excitation wavelength of 488 nm and ImaGene software (BioDiscovery, Inc.) to quantify fluorescence. Data are reported as the average RFU of four of six replicates (after removal of highest and lowest values) for each glycan represented on the array.

2.10. FGF binding to HEK293T cells

HEK293T cells were cultured to ~75% confluency. To assay FGF binding to its receptor, medium was completely replaced with fresh medium containing 10% fetal calf serum and 1 mM Na_3VO_4 for 30 min. PolyLacs purified as described above were added to the monolayer at 1 mg/ml and incubated for 10 min. Recombinant b-FGF (Sigma) was then added to a final concentration of 25 ng/ml and incubated for 5 min. Cells were then washed three times with TBS. Cell lysates were prepared with lysis buffer provided in the b-FGF ELISA kit (Ray Biotech), followed by centrifugation. Each lysate containing 100 mg protein was subjected to an ELISA inhibition assay to determine the amount of FGF bound to cells in the presence or absence of polyLacs.

2.11. Immunoprecipitation and immunoblotting

HEK293T cells were cultured in medium supplemented with 10% fetal calf serum. Na_3VO_4 was added to a final concentration of 1 mM and incubated for 30 min. FGF was added to a final concentration of 25 ng/ml in the presence or absence of polyLacs (1 mg/ml) and incubated for 5 min. Cells were collected using a rubber policeman, washed with cold TBS containing 1 mM Na_3VO_4 , and lysed with 1% NP-40 in TBS. Each lysate was pre-cleared with 20 μl protein A/G agarose beads at 4 °C for 20 h. Protein A/G beads (20 μl) were then coated with anti-FGFR2 antibody (2 μg , rabbit polyclonal, GenTex) in 500 μl TBST at 4 °C for 20 h. Pre-cleared lysates were reacted with antibody-coated beads at 4 °C for 20 h. Beads were washed three times with TBST and bead-bound materials were eluted by boiling in SDS buffer for 3 min. Proteins were resolved on 4–12% gradient SDS–polyacrylamide gels and transferred to an immobilon-P filter (Millipore). After blocking with Odyssey blocking buffer (Li-Cor Biosciences), the filter was reacted with diluted (1:500) anti-tyrosine phosphate antibody (4G10, Upstate Biologicals) in blocking buffer containing 0.1% Tween 20 for 60 min, followed by incubation of diluted (1:5000) IRDye-conjugated anti-mouse IgG antibody (Li-Cor Biosciences) for 45 min. After washing with TBST, the filter was scanned by the Odyssey imaging system to detect bands.

2.12. Immunofluorescence microscopy

Semen was smeared on glass slide glasses, air dried and fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. Sperm cells and seminal fluid on the slide glass were reacted with diluted (1:100) anti-FGF2 antibody (rabbit polyclonal,

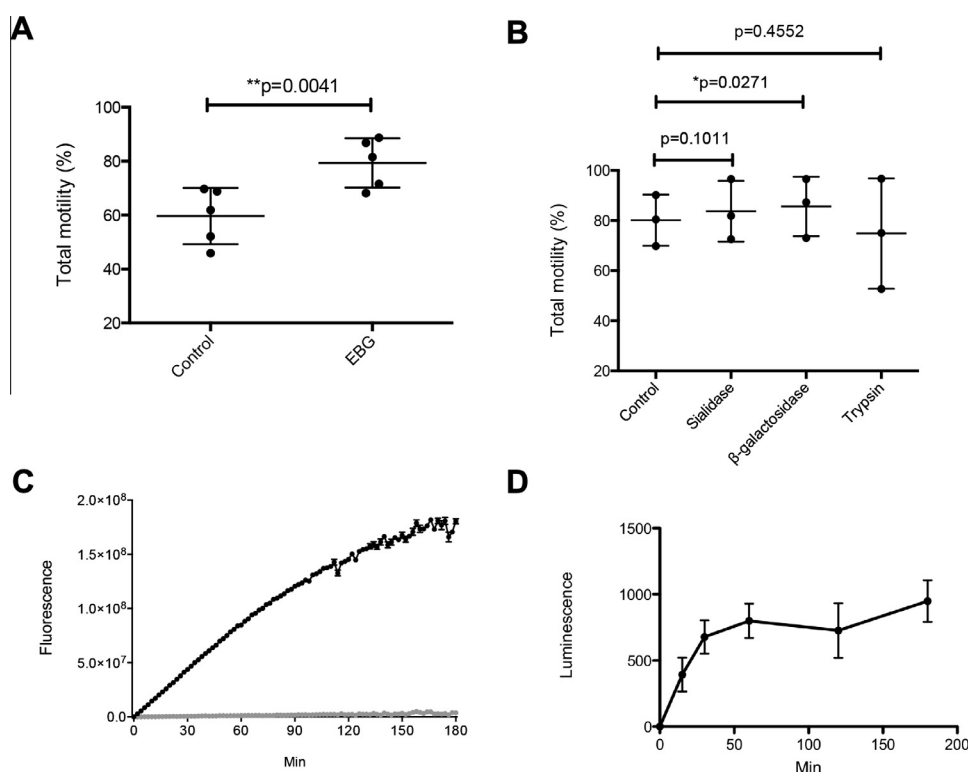


Fig. 1. Effect of EBG treatment on human sperm. (A) Effect of EBG on sperm motility. (B) Effect of other enzymes on sperm motility. In (A) and (B), spermatozoa from a volunteer were treated with (plus) or without (minus) EBG, 50 $\mu\text{Units/ml}$, at room temperature for 15 min before analysis. Statistical analysis was via a unpaired 2-tailed *t*-test. (C) Effect of EBG on calcium levels measured by fluorescence in human spermatozoa incubated with (black) or without (grey) EBG. The average value of triplicate data points are shown. (D) Levels of cAMP in human spermatozoa incubated with or without EBG. Sperm cells were subjected to protein kinase C-based cAMP measurement. Luminescence values are values from EBG-treated cells minus values from buffer-treated controls over the test period. Each point represents an average of ten readings.

rabbit IgG) and mouse monoclonal anti-LeY (clone AH6, mouse IgM) for 1 h. Control slide received no primary antibody. After washing with PBS, sample was reacted with A568-labeled anti-rabbit IgG antibody and A488-labeled anti-mouse IgM antibody. After washing with PBS, slides were mounted with Vectashield (Vector) with DAPI.

3. Results

3.1. Effect of EBG on human sperm motility

We analyzed human sperm motility using computer-assisted sperm motility analyzer. This analysis showed that EBG enhanced sperm motility, whereas sperm treated with sialidase and with β -galactosidase showed motility comparable to untreated sperm (Fig. 1A and B). Treatment with trypsin did not enhance sperm motility, excluding the possibility that the observed EBG effect is non-specific.

Sperm tail motility reportedly requires ATPase and ion channel activities [31–33]. To begin to analyze these parameters, we measured calcium levels in sperm and observed that they were significantly increased in EBG-treated sperm cells (Fig. 1C). Since calcium flux into sperm is mediated by cyclic nucleotide-dependent gated channels [31], we examined cAMP levels and found that they were indeed enhanced in EBG-treated sperm cells (Fig. 1D and Fig. S1). These results suggested that polyLacs attached to the sperm tail suppressed sperm motility through a cAMP- and calcium channel-dependent mechanism.

3.2. Isolation and characterization of sperm-associated polyLacs

As a previous study of human sperm glycans did not detect polyLacs [34], we hypothesized that polyLacs are not an intrinsic component of human sperm but are derived from seminal fluid and associated with the tail. To characterize sperm-associated polyLacs, we isolated sperm cells from semen washed twice with PBS. Sperm cells were then digested with protease K, and water-soluble materials were fractionated by gel filtration. This analysis revealed the existence of large carbohydrates, presumably polyLacs (Fig. 2A-a). Following EBG treatment, a substantial portion of large carbohydrates was converted to smaller fragments, demonstrating that they are indeed polyLacs (Fig. 2A-b).

MALDI-MS analysis of sperm-associated polyLacs revealed the presence of sulfated, sialylated, fucosylated Hex–HexNAc units (Fig. 2B). Notably, the presence of blood group H antigen, LeX antigen and LeY antigen was evident based on both composition and additional MS/MS analysis (Table S1). In particular, in-source prompt fragmentation yielded oxonium ions at m/z 638 and 812, corresponding to LacNAc with 1 and 2 Fuc, respectively. Furthermore, glycan fragments with compositions such as 3 LacNAc and 4 Fuc with and without additional sulfate were detected. Thus the internal portion of polyLacs was highly substituted with fucose, in addition to sialylation and sulfation seen at the termini.

To determine the origin of sperm-associated polyLacs, we performed immunohistochemistry of human tissue from organs functioning in spermatogenesis using anti-Lewis Y antibody in tissues

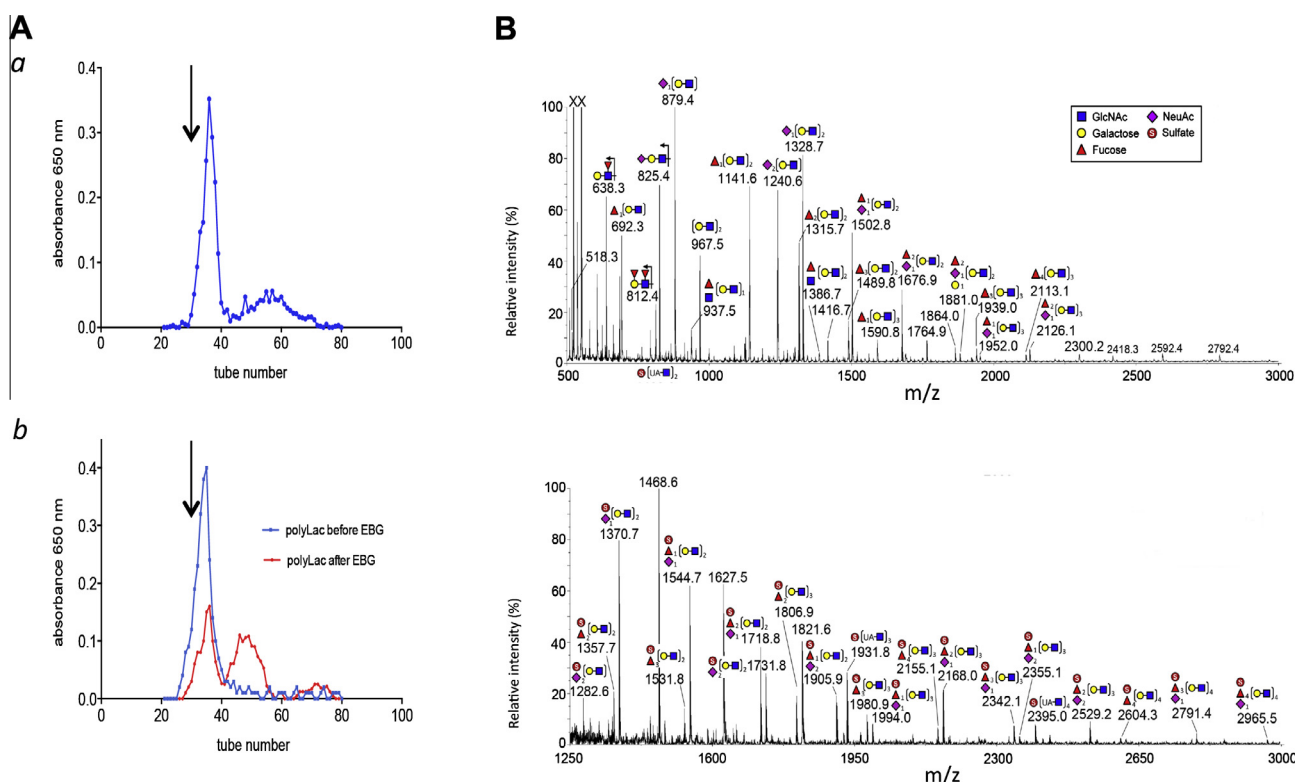


Fig. 2. Structure and localization of sperm-associated large glycans. (A) Sephadex G-50 gel filtration chromatograms of seminal fluid polyLacs. (a) PolyLacs eluted between tube number 31 and 39 were collected after gel filtration of a mixture of glycopeptides prepared from pooled seminal fluid. (b) Seminal fluid polyLacs before (blue) and after (red) EBG digestion. Note that large glycopeptides were digested to fragments eluted at tube numbers 42–56 and 64–77. Each fraction was monitored for hexose by an anthrone color reaction [28]. Arrows show the elution position of blue dextran or void volume. (B) MALDI-TOF mass spectrometry of non-sulfated (upper) and sulfated (lower) glycan fragments afforded by direct permethylation of the same sample, as detected in positive and negative ion modes as $[M+Na]^+$ and $[M-H]^-$, respectively. In addition to the same series of variably fucosylated [Hex–HexNAc]_{1–3} glycan fragments, oxonium ions corresponding to terminal H/LeX (m/z 638), LeY (m/z 812) and sialylLacNAc (m/z 825) were detected. In negative ion mode, sulfated and fucosylated [Hex–HexNAc]_{1–4} fragments with and without sialylation were identified. All major peaks were labeled and annotated using the symbol set shown.

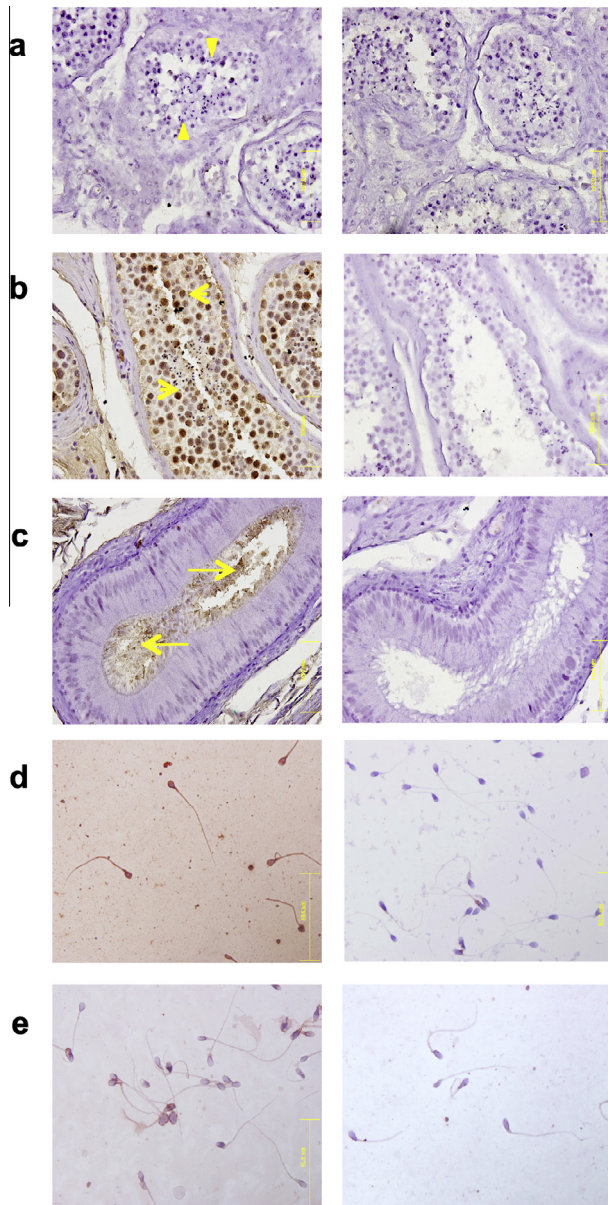


Fig. 3. Immunohistochemistry of tissue sections and sperm smears using anti-Lewis Y antibody. (a) Testis; (b) epididymis; (c) ductus deferens; (d) human semen; (e) PBS-washed sperm. Tissue sections and semen or sperm smeared slides were treated with (right) or without (left) EBG and immunostained by anti-Lewis Y antibody. Note that spermatogenic cells in testis (arrowheads in (a)) are not stained, whereas epithelial cells of ductuli epididymis (short arrows in (b)) and mucus in ductuli efferentis (long arrows in (c)) were stained positively with the antibody. Scale bars: 100 µm (a–c), 50 µm (d and e).

treated with or without EBG. While testis was negative for LeY antigen (Fig. 3a), LeY antigen was seen in epithelial cells of tissue from epididymis, which contains spermatozoa newly released from testis (Fig. 3b) and from ductus deferens, which contains fully matured spermatozoa (Fig. 3c). A semen smear, which contains a mixture of sperm cells and seminal fluid, was positive for LeY antigen (Fig. 3d), but LeY antigen was lost from these tissue sections after EBG treatment (Fig. 3c and d, right panels). A twice-washed spermatozoa smear treated with EBG showed loss of LeY positivity (Fig. 3e). These results strongly suggest that LeY antigenic polyLacs attached to mature sperm cells were secreted from epithelial cells of the epididymis and/or ductus deferens into seminal fluid.

3.3. Binding of polyLacs to FGF

Increased cAMP levels seen in EBG-treated sperm (Fig. 1D) suggested a role for FGF signaling in the process, since FGF activates the FGFR to increase cAMP levels through the PLC γ /Ca $^{2+}$ /PKC pathway [35]. We tested polyLac binding to FGFs by a plate assay, using plates coated either by recombinant FGF1 or FGF2. Seminal fluid polyLac bound to both FGF1 and FGF2 (Fig. 4A and B), whereas the same polyLacs did not bind to epidermal growth factor (Fig. 4B). Glycan array analysis confirmed FGF2 binding to sulfated glycans and fucosylated and non-fucosylated polyLac structures (Fig. 4C). Immunofluorescence microscopy of human semen showed co-localization of FGF2 and LeY (Fig. S2).

It is known that FGFs interact with heparin and/or heparan sulfate, which is essential for FGFR binding and subsequent signaling [36,37]. We asked if polyLacs bind to FGF at the heparin-binding site, by testing the effect of heparin on polyLac binding to FGF by plate assay (Fig. 4D). Results clearly showed that heparin has no inhibitory effect of polyLac binding to FGF1 and FGF2.

3.4. Effect of polyLacs on FGF signaling

The results described above suggest that FGF binds to polyLacs, potentially hindering FGF binding to cell surface receptors and

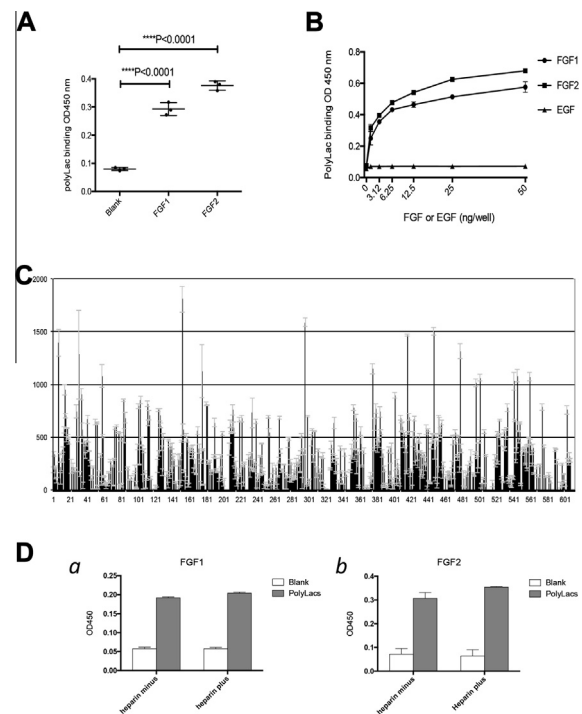


Fig. 4. Binding of seminal fluid polyLacs to FGF. (A) Plastic plates were coated with recombinant human FGF1 or FGF2 prior to addition of polyLacs. PolyLacs bound to each well were assessed by anti-LeY antibody (AH6, mouse IgM) followed by peroxidase-conjugated anti-mouse IgM antibody and analysis using a peroxidase color reaction. (B) Dose-dependent binding of seminal fluid polyLacs to FGF1, FGF2, and epidermal growth factor (EGF). (C) Top 10 strongest binders are: 152, Gal β 1-4(Fuc α 1-3)GlcNAc β -Sp8; 296, 4S(3S)Gal β 1-4GlcNAc β -Sp0; 447, Gal β 1-4(Fuc α 1-3)GlcNAc β 1-6GalNAc-Sp14; 416, Fuc α 1-2Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3GalNAc α -Sp14; 7, Fuc α -Sp9; 478, Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-2Man α -Sp0; 31, (3S)Gal β 1-3GlcNAc β -Sp8; 375, Fuc α 1-4(Fuc α 1-2Gal β 1-3)GlcNAc β 1-2Man α 1-3(Fuc α 1-4(Fuc α 1-2Gal β 1-3)GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc β -Sp19; 175, GlcNAc β 1-2Gal β 1-3GalNAc α -Sp8; 58, Fuc α 1-2Gal β 1-3GalNAc β 1-3Gal α -Sp9; 545, GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-2Man α 1-6(GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc β -Sp12. (D) Effect of heparin on polyLac binding to FGFs. PolyLac binding to FGF1 (a) and FGF2 (b) in the presence or absence of heparin at 100 µg/ml.

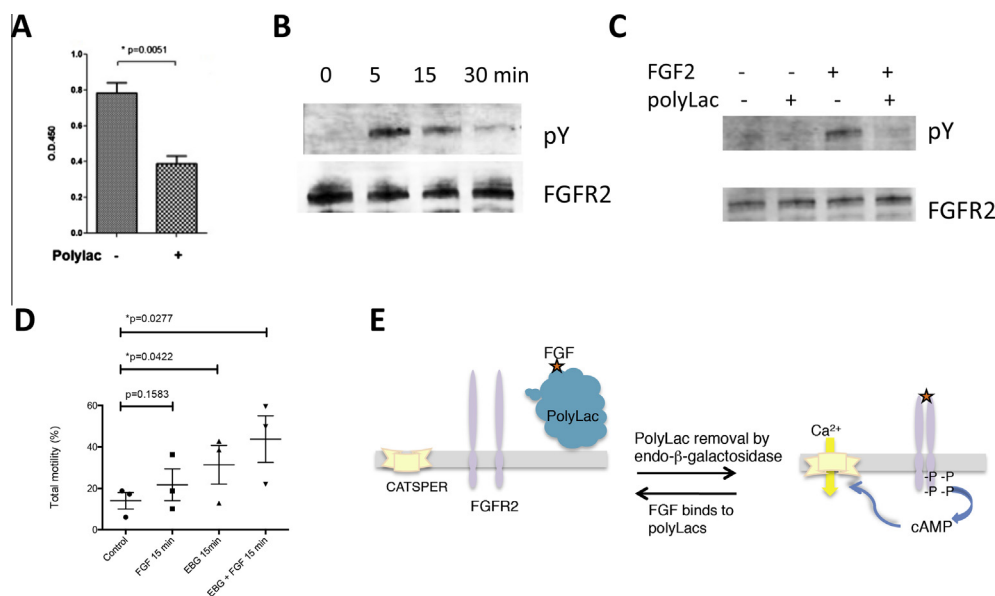


Fig. 5. Effect of polyLacs on FGF signaling in HEK293T cells. (A) Binding of FGF to HEK293T cells in the presence or absence of polyLacs. FGF was measured by FGF-ELISA inhibition assay. (B) Time course of FGFR2 tyrosine phosphorylation in response to addition of FGF to cells. (C) Effect of seminal fluid polyLacs on tyrosine phosphorylation of FGFR2. In (B) and (C), cells were treated with FGF in the presence or absence of polyLacs, and lysates were immunoprecipitated with anti-FGFR2 antibody and immunoblotted for phospho-tyrosine (upper panel). The filter was then stripped and immunoblotted for FGFR2 (lower panel). (D) Effect of FGF on motility of EBG-treated human sperm. Sperm samples from three individuals were tested. Statistical analysis was performed using a paired, one-tailed *t*-test. (E) Proposed mechanism for polyLac-dependent regulation of sperm motility. Human sperm tails express the receptor tyrosine kinase FGFR2 and CATSPER1, a cAMP-dependent calcium channel. FGF binds to polyLacs near the sperm tail surface, which hinders FGF binding to the receptor and result in attenuation of sperm motility (left). Once polyLacs are removed by EBG digestion, FGF binds to FGFR2 and stimulates tyrosine phosphorylation, resulting in PLC γ and PKC signaling [39], calcium flux through cyclic nucleotide-gated channels, and enhanced sperm motility (right).

attenuating FGF signaling. Since polyLacs and FGF are expressed in many cell types, we asked whether this effect occurs in other cell types. To do so, we employed HEK293T cells, since a publicly available gene expression database indicates that this line expresses high levels of FGFR2 mRNA. Binding of FGF to HEK293T cells was significantly higher in the absence of seminal fluid polyLacs compared to binding in the presence of polyLacs (Fig. 5A), suggesting that these glycans block FGF binding to the receptor. Moreover, following addition of FGF to HEK293T cells, FGFR2 became tyrosine-phosphorylated, with activity peaking in 5 min (Fig. 5B), whereas FGFR2 tyrosine phosphorylation at that time point was inhibited by seminal fluid polyLacs (Fig. 5C).

Above described results suggest a possibility that removal of polyLacs and supplementing FGF increases FGF signaling. We tested this hypothesis by comparing human sperm motility following treatment with EBG, FGF or both. EBG or FGF treatment increased motility relative to untreated control sperm, but the highest motility was observed in sperm treated with both (Fig. 5D).

4. Discussion

Here, we show that human sperm tail is heavily covered by fucosylated, sialylated and sulfated polyLacs. EBG treatment removed these glycans from the sperm tail, leading to increased motility, calcium influx and increased cAMP levels (Fig. 1). Therefore we propose that mechanisms underlying sperm motility require cAMP signaling, which potentially targets sperm-specific calcium transporters, such as CatSpers [38,39] (Fig. 5E).

Several studies have reported structures of glycans isolated from seminal fluid glycoproteins, including α_1 -acid glycoprotein [40], clusterin, galectin-3-binding glycoprotein, prostatic acid phosphatase, protein C inhibitor [41], and glycodefin [42,43]. However, polyLacs were not found in these glycoproteins. It is likely that the polyLacs characterized here are carried by a specific carrier protein, which have yet to identify. Identification of a seminal fluid

glycoprotein carrying polyLacs may provide important insight into regulation of sperm motility.

We showed in this study that FGFs bind to complex glycans of which structures are shared with seminal fluid polyLacs (Fig. 4C). It is known that heparin and heparan sulfate bind to both FGF and FGFR, which promotes the ligand-receptor binding and tyrosine phosphorylation of FGFR [35,37]. However, our binding assays, including the glycan array, did not demonstrate polyLac binding to FGFR2R2 (data not shown). By contrast, polyLacs bound to FGFs (Fig. 4). Interestingly, heparin did not inhibit polyLac binding to FGFs (Fig. 4D), suggesting that polyLacs binds to FGF at the site distinct from heparin binding site. Furthermore, it appears that binding of polyLacs to FGFs was enhanced in the presence of heparin. This effect was clearly demonstrated by binding of sulfated polyLac, keratin sulfate, to FGF2: binding of keratin sulfate was elevated in the presence of heparin (Fig. S3). These observations not only exclude a possibility that polyLacs attenuate FGF signaling by inhibiting heparin binding to FGF, but also suggest a possibility that heparin-bound FGF changes its conformation to be favorable for polyLac binding.

We show here that tyrosine phosphorylation of FGFR2 in HEK293T cells grown under normal tissue culture conditions is down-regulated by seminal fluid polyLacs (Fig. 5A–C). This finding suggests that polyLac-mediated regulation of FGF occurs in a variety of tissue types. Thus, we conclude that sperm cells employ a conserved mechanism requiring highly concentrated polyLacs to regulate motility.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.febslet.2013.07.056>.

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